

Development and Application of Reference Antisera against 15 Hemagglutinin Subtypes of Influenza Virus by DNA Vaccination of Chickens

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Reference antisera were produced against 15 influenza hemagglutinin (HA) subtypes using DNA vaccination to produce a high-quality polyclonal serum to the HA protein without antibodies to other influenza viral proteins. The HA gene from each of 15 different HA subtypes of influenza virus was cloned into a eukaryotic expression vector and injected intramuscularly, together with a cationic lipid, into 3- to 4-week-old specific-pathogen-free chickens. Birds were boosted twice at 4-week intervals after the initial injection, and in general, antibody titers increased after each boost. The antisera were successfully applied in the hemagglutination inhibition test, which is the standard method for the classification of the HA subtypes of influenza virus. We also demonstrated the HA specificity of the antisera by Western blot and immunodot blot analysis. DNA vaccination also provides a safer alternative for the production of HA-specific antibodies, since it is produced without the use of live virus.

Type A influenza viruses infect a wide variety of hosts, but the greatest diversity of viruses is seen in birds (6, 18). Type A influenza viruses are serologically divided into 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes, including a recently described novel HA subtype that was obtained from black-headed gulls (2). Wild waterfowl and shorebirds provide a reservoir for all 16 influenza HA subtypes, and most infections are subclinical in these species. However, in poultry, influenza virus infections cause a wide range of disease signs, and the features of infection are variable depending on virus strain, host species, host age, concurrent infections, etc. In poultry, the highly pathogenic form of avian influenza (AI) is usually associated with a multiorgan systemic disease accompanied by high morbidity and mortality. Highly pathogenic AI (HPAI) is a World Organization for Animal Health listed disease and is subject to international reporting. Of the 16 HA subtypes that have been identified, only strains within the H5 and H7 subtypes cause the HPAI form of the disease, and therefore, these subtypes present a much greater concern.

The characterization of newly isolated influenza viruses from poultry is an important step in developing an appropriate regulatory response. Virus characterization involves subtype determination of the two surface proteins, HA and NA, and assessment of pathogenicity using *in vitro* and *in vivo* assays (18). The hemagglutination inhibition (HI) test using reference antisera is the standard method used to subtype the HA of influenza A viruses (12, 17). The basis of the HI test is that influenza viruses will hemagglutinate erythrocytes through the interaction of sialic acid and sialic acid receptors on the HA protein. Since the influenza virion can attach to more than one

erythrocyte at a time, this allows for cross-linking or clumping of erythrocytes by the virus. This hemagglutination can be inhibited by antibodies directed against the HA protein. Antibodies to the HA are subtype specific, so that antibodies against one subtype will not typically react with another subtype. Therefore, the HI test has been used as the primary and classical method of identifying the HA subtype of an unknown virus. The common means of producing reference antisera for AI viruses is by injecting chickens with live or killed whole-virus preparations. This procedure produces antibodies to the HA protein that are useful for HI tests; however, it also stimulates the production of antibodies to other influenza viral proteins including the NA protein, which can interfere with HI test results. Furthermore, laboratories must be equipped with adequate biosafety facilities to safely work with live viruses in birds. This is especially important when dealing with HPAI viruses or viruses that have the potential to cause disease in humans.

Several experimental studies have demonstrated the ability of DNA vaccines to elicit protective immune responses in different hosts, including chickens (3, 5). DNA vaccination can produce both cellular and humoral immune responses that are similar to those of live virus infection or vaccination (20). In our previous study, DNA vaccination could produce a measurable and protective antibody response in chickens (16). Although DNA vaccines need to be more efficacious and less expensive to produce for them to be practical as a vaccine for commercial poultry, DNA immunization can be applied and used for diagnostic and research purposes. In this study, we applied DNA vaccination to prepare reference antisera against 15 HA subtypes of influenza virus. The advantage of preparing antisera by DNA vaccination and the potential applications of the antisera are described.

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TABLE 1. Strains, EEV constructs, and HI responses after DNA vaccination

Strain	Subtype	EEV construct	Mean log ₂ HI titer ± SD ^b			No. of responses/ total no.
			First (4 wk)	Second (8 wk)	Third (12 wk)	
A/chicken/AR/30402/99	H1N1	H1-pCIneo	2.0 ± 0.8	6.0 ± 0.8	9.3 ± 1.5	6/7
A/herring Gull/DE/677/88	H2N8	H2-pCIneo	1.6 ± 1.3	6.3 ± 1.5	7.1 ± 1.7	7/7
A/duck/NY/13822/95	H3N8	H3-pCIneo	2.1 ± 1.3	4.8 ± 3.0	7.6 ± 2.3	7/7
A/shoveller/Buryatia/1898/00	H4N? ^d	H4-pCIneo	1.3 ± 0.5	5.6 ± 1.9	8.0 ± 1.5	7/7
A/turkey/WI/68	H5N9	H5na-pCIneo	5.7 ± 1.8	8.8 ± 1.8	10.2 ± 1.2	8/8
A/chicken/Scotland/59	H5N1	H5ea-pCIneo	2.9 ± 1.2	6.3 ± 1.3	6.9 ± 0.7	7/7
A/chicken/NY/13237-6/98	H6N8	H6-pCIneo	3.0 ± 1.5	7.3 ± 1.0	8.1 ± 1.1	8/8
A/turkey/NY/4450/94	H7N2	H7na-pCIneo	5.1 ± 0.8	6.7 ± 1.6	9.0 ± 1.9	8/8
A/chicken/IT/1067/99	H7N1	H7ea-pCIneo	4.5 ± 0.5	5.5 ± 1.4	6.8 ± 1.3	7/7
A/turkey/Ontario/6118/67	H8N4	H8-pCIneo	5.1 ± 1.6	9.1 ± 0.7	10.9 ± 1.2	7/7
A/chicken/Korea/96008/96	H9N2	H9-pCIneo	3.9 ± 1.6	7.7 ± 1.0	8.0 ± 1.2	7/7
A/quail/NJ/25254/95	H10N7	H10-pCIneo	2.0 ± 1.9	3.7 ± 2.6	6.2 ± 1.7	7/7
A/chicken/NJ/15906/96	H11N1	H11-pCIneo	1.6 ± 0.8	3.3 ± 2.1	6.7 ± 2.3	7/7
A/duck/Alberta/60/76	H12N5	H12-pCIneo	1.0 ± 0.0	2.6 ± 2.1	7.4 ± 0.5	5/7
A/gull/MN/945/80	H13N6	H13-pCIneo	3.0 ± 2.8	4.3 ± 2.8	6.7 ± 0.6	4/8
A/mallard/Gurjev/263/82	H14N5	H14-pCR3.1	0.0 ± 0.0	1.0 ± 0.0	4.0 ± 0.0	1/8 ^a
A/shearwater/W.A./2576/79 ^c	H15N9	H15-pCIneo	2.0 ± 0.0	6.0 ± 1.3	6.8 ± 0.8	8/8

^a This result was derived from using a higher dose of DNA vaccine (200 µg/dose).

^b First, second, and third refer to first, second, and third vaccinations.

^c W.A., West Australia.

^d Subtype not determined.

2002, The University of Georgia, Athens, Georgia, and were published as a proceedings manuscript [9a].)

MATERIALS AND METHODS

Construction of DNA vaccine. Eukaryotic expression vectors (EEVs) expressing 1 of 15 different subtypes of HA protein derived from 17 strains of influenza viruses were prepared as previously described (Table 1 and Fig. 1) (8). Briefly, the full coding sequences of the HA genes were amplified by reverse transcription-PCR and cloned into the pAMP plasmid vectors (Life Technologies, Gaithersburg, MD). The HA genes were then cut with appropriate restriction enzymes and directionally subcloned into the pCIneo (Promega, Madison, WI) EEV, which contains the cytomegalovirus early promoter. The reverse transcription-PCR product of an H14 gene was also cloned directly into the pCR3.1 EEV (Invitrogen, Carlsbad, CA) according to the manufacturers' protocol. The coding sequence of each HA gene insert was confirmed by sequencing. Furthermore, the expression of the inserted sequence was tested in vitro using African green monkey kidney (Vero) cells by indirect immunofluorescence staining as described previously (8). Briefly, 90% confluent Vero cells were transfected with 1 µg of each EEV using Lipofectamine 2000 (Invitrogen). After 24 h of incubation at 37°C, cells were fixed with 2% paraformaldehyde (Sigma, St. Louis, MO) and reacted with antisera from chickens immunized with inactivated influenza virus as described below. Cells were observed for fluorescence after fluorescein isothiocyanate-conjugated anti-chicken immunoglobulin G (IgG) (Sigma) was added.

DNA vaccination in chickens. For the vaccine inoculum, 20 µl of Lipofectin (Invitrogen) adjuvant was mixed with 100 µl of phosphate-buffered saline and incubated for 30 min at room temperature (RT) to form liposome. One hundred micrograms of the plasmid DNA was diluted into a total volume of 280 µl phosphate-buffered saline and mixed with the liposome. The plasmid-liposome mixture was allowed to equilibrate 15 min before inoculation. Each test group had seven or eight 3-week-old specific-pathogen-free (SPF) White Rock chickens, which were maintained at Southeast Poultry Research Laboratory, Athens, Georgia. Each bird received two separate injections of 0.2 ml intramuscularly into each breast muscle. Birds were boosted twice at 4-week intervals. The birds were bled to determine HI antibody titers before each booster inoculation and at the end of the experiment. To determine the weekly HI immune response of chickens to a DNA vaccine, one additional group of eight birds was vaccinated with H5-specific EEV and bled weekly for serum collection.

Production of reference antisera by conventional methods. Fifteen different subtypes of influenza virus were grown in 10-day-old embryonating chicken eggs, and the infectious allantoic fluid was pooled for each virus. The strains used for production of oil emulsion vaccine are not exactly the same as the strains used for the DNA vaccine constructs. Infectious virus was inactivated by treatment with

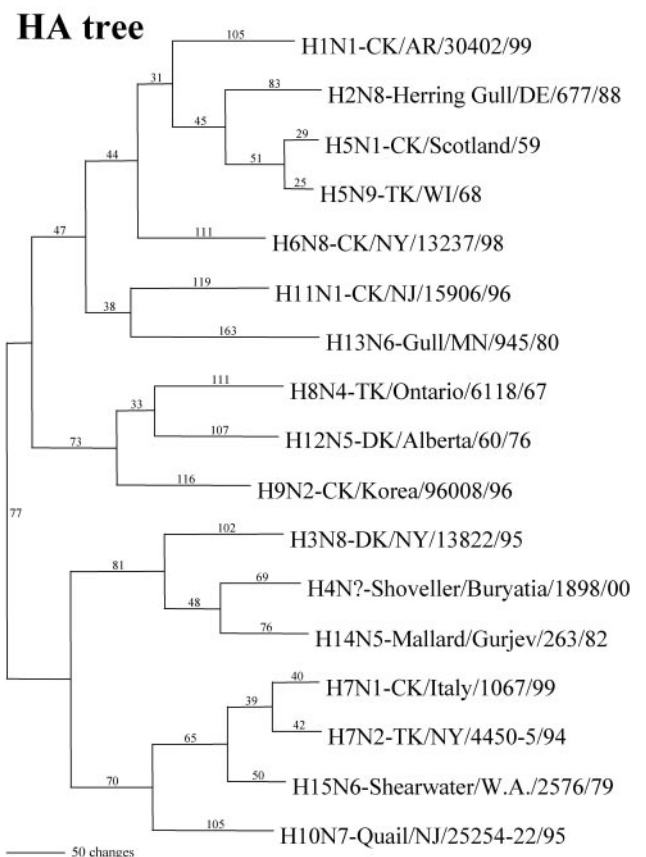


FIG. 1. Phylogenetic tree based on amino acid sequences of the HA gene. The tree was generated by the maximum-parsimony method with the PAUP4.0b10 program and is the result of a heuristic search and midpoint rooting. Branch lengths are included in the tree. Abbreviations: CK, chicken; DK, duck; TK, turkey; W.A., West Australia. Standard two-letter abbreviations are used for states in the United States.

betapropiolactone (Sigma) at a final concentration of 0.1%. The inactivated virus was used to produce an oil emulsion vaccine as previously described (15). Three-week-old SPF chickens were inoculated subcutaneously with the inactivated oil emulsion vaccines. Three weeks later, the chickens were booster vaccinated by the same route with the same amount of vaccine. Two weeks after the booster vaccination, birds were bled to collect serum. Viruses used in this experiment were obtained from the SEPRL AI virus repository and included the following strains: A/chicken/NY/14862-3/94 (H1N1), A/gull/MD/19/77 (H2N9), A/turkey/England/69 (H3N2), A/chicken/AL/75 (H4N8), A/turkey/WI/68 (H5N9), A/turkey/MA/65 (H6N2), A/turkey/OR/71 (H7N3), A/turkey/Ontario/6228/67 (H8N4), A/turkey/WI/66 (H9N2), A/chicken/Germany/N/49 (H10N7), A/duck/Memphis/5446/74 (H11N9), A/duck/Alberta/60/76 (H12N5), A/gull/MD/704/77 (H13N6), A/mallard/Gurjev/263/82 (H14N5), and A/shearwater/West Australia/2576/79 (H15N9).

HI test. Serum from DNA- and inactivated virus-vaccinated birds were tested for HA subtype-specific antibodies with the HI test (17). Influenza viruses representing all 15 subtypes, which were used for the DNA vaccination, except the H4 subtype, were used as antigens. Because the A/shoveller/Buryatia/1898/00 virus was not available (we used an HA cDNA clone of this virus to make a DNA vaccine), we instead used A/duck/NJ/5406-27/94 (H4N6) virus as an antigen. The HI test was a standard beta test, using 4 HA units of antigen in 96-well plates, where the test serum had been diluted twofold. HI endpoint titers were determined as the reciprocal of the highest serum dilution that produced complete inhibition of hemagglutinating activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot assays. Infectious allantoic fluid of H5 (A/turkey/WI/68) virus was denatured in sample buffer (Pierce, Rockford, IL) and boiled for 5 min. Denatured proteins were separated in a 10 to 20% polyacrylamide gel gradient (Cambrex, Rutherford, NJ) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 35 mA for 60 min. The proteins were then transferred onto a nitrocellulose (NC) membrane (Bio-Rad, Hercules, CA), and the blots were blocked with 2% (wt/vol) nonfat skim milk for 2 h at room temperature. H5 subtype-specific antiserum, produced by conventional or DNA vaccination, was diluted 1:200 and 1:500, respectively, incubated for 1 h at RT, and then washed three times in washing solution (KPL, Gaithersburg, MD). The secondary antibody goat anti-chicken IgG (KPL) was added (1:1,000), and the mixture was incubated for 1 h at RT. Following the washing step described above, the membrane was developed with the chromogen/substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (KPL) for 15 min at RT. The reaction was stopped by rinsing with distilled water.

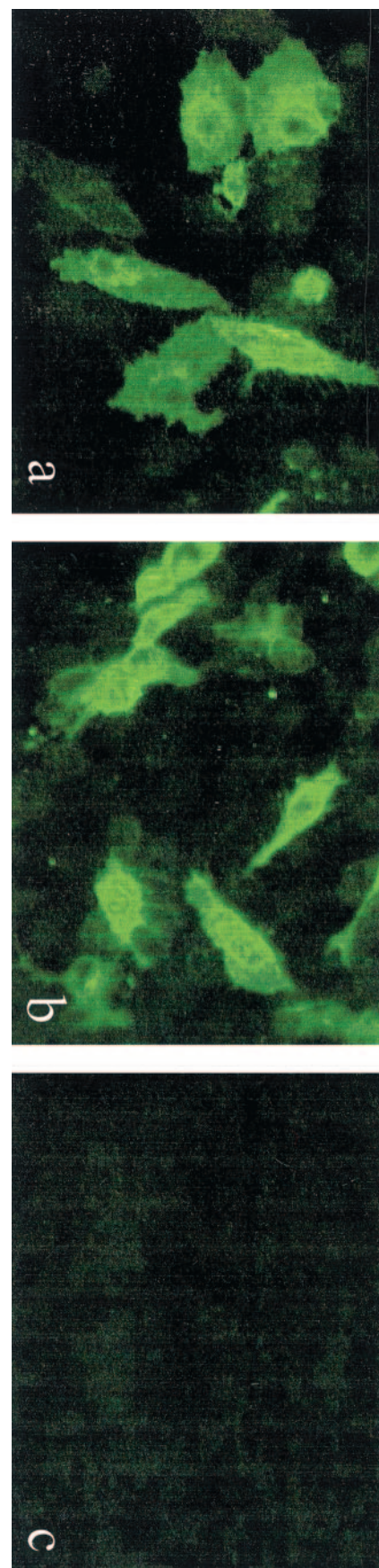
Immunodot blot assay. An immunodot blot assay was conducted as described previously (7), with the following modifications. One hundred microliters of allantoic fluid was dotted onto a 0.45- μ m NC membrane using the 96-well Hybridot manifold (BRL, Gaithersburg, MD). The strains used for this assay were the same as those used for the HI assay. After vacuum blotting, the membrane was blocked with 2% (wt/vol) nonfat milk in TST (10 mM Tris, 0.15 M NaCl, 0.05% Tween 20) at 37°C for 30 min. It was then reacted with diluted antibody (1:100) in TST at RT for 1 h. After the membrane was washed with three changes of TST, phosphatase-labeled goat anti-chicken IgG (Sigma) (1:500 dilution) was added and incubated for 2 h at RT. The preparations were then developed by using a nitroblue tetrazolium/BCIP kit (KPL).

RESULTS

Preparation of DNA vaccine. The complete coding sequence of the HA genes from 17 different influenza viruses were cloned into pCIneo EEVs. The H14 HA gene was cloned into the pCR3.1 vector in addition to the pCIneo vector (Table 1). The sequences of the HA inserts were confirmed as reported above. The phylogenetic relationship of the HA genes of the isolates used in this study is shown in Fig. 1. The 15 HA subtypes showed 34.7 to 81.0% amino acid identity with each other and were divided into different clusters as described previously (1). H7 and H15 subtypes shared the highest amino acid identity (81.0%) among subtypes. More than 75% amino acid identity was also observed between H2 and H5 (77.0%) and between H4 and H14 (78.2%).

To confirm that each EEV expressed the HA gene that was inserted, the plasmids were transfected into Vero cells and

FIG. 2. Indirect immunofluorescent antibody assay. Vero cells were transfected with H5-pCIneo (a) or H14-pCR3.1 EEV (b) and reacted with H5 and H14 antisera, respectively, obtained by a conventional method. A nontransfected negative control (c) was also included and reacted with both antisera. Bright fluorescence in the cytoplasm was observed in both EEV-transfected cells (a and b). Magnification, $\times 200$.



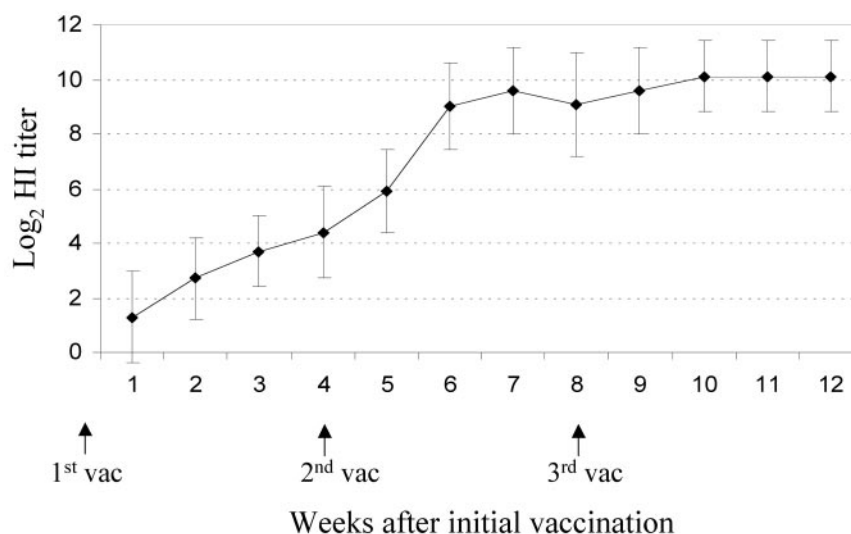


FIG. 3. Eight 3-week-old SPF birds were vaccinated with H5-pCIneo and booster vaccinated at 4 weeks and 8 weeks after initial vaccination (vac). Sera were collected from individual birds every week (1 week to 12 weeks), and the HI titers were determined. Mean \log_2 HI titers and standard deviations are indicated in the graph generated by the Excel (Microsoft) program.

tested by an indirect fluorescent antibody assay for expression of the HA protein. All constructs had detectable immunofluorescence in the cytoplasm of the transfected cells, and H5- and H14-specific EEV data are shown in Fig. 2.

Weekly immune response of chickens against the H5 DNA vaccine. To examine the course of the immune response to the DNA vaccine, H5-specific EEV was used to vaccinate 3-week-old SPF chickens, and serum was collected weekly from individual birds to determine the HI titer (Fig. 3). During the first 4 weeks following a single vaccination, the HI titer of the antibody gradually increased every week (0 to 4.4 \log_2 HI titer). After the booster injection, which was 4 weeks following the initial vaccination, approximately a 32-fold increase was observed within the first 2 weeks (9.0 \log_2 HI titer), but the HI titer remained relatively steady after 2 weeks. After the third vaccination, a less-than-fourfold increase in titer (10.1 \log_2 HI titer) was observed during the first 2 weeks, but the HI titer remained steady after that point.

Immune response to the 15 HA subtype-specific DNA vaccines. In each of the groups of birds vaccinated with the 15 different EEVs, all or most birds produced measurable antibody responses. However, H13-pCIneo generated only four seropositive birds out of eight vaccinated birds, and H14-pCIneo did not produce any seropositive birds. We also tested H14-pCR 3.1 EEV, and this DNA vaccine also did not induce a detectable immune response when the same amount of the DNA was inoculated (data not shown). We were able to get a low antibody titer from one bird out of eight vaccinated birds when the H14-pCR3.1 plasmid concentration of inoculum was increased to 200 μg per bird (Table 1).

The highest antibody responses were observed in chickens vaccinated with EEVs that had HA inserts derived from the H1, H5, H7, or H8 subtype. After the third vaccination, the reciprocal HI titers for the chickens in these groups were greater than 1:512. Moderate HI responses were observed in other groups vaccinated with H2, H3, H4, H6, H9, H10, H11, H12, H13, and H15 EEVs, with a range of 1:64 to 1:512.

Cross-HI test. To determine the subtype specificity of the antibodies produced by DNA vaccination, the HI test was conducted with antigen prepared from 15 subtypes of influenza virus. Antisera to the 15 subtypes made by the conventional method were also included in the test for comparison. In general, the HI test with sera prepared by DNA vaccination or the conventional method produced similar results (Tables 2 and 3). Usually, cross-reaction with heterologous antigen was negligible ($<1:4$). However, H15 antigen and H7 subtype antisera prepared by both methods exhibited high cross-reactivity (1:8 to 1:16). This was somewhat expected because of the high sequence relatedness between these two strains (Fig. 1). We also observed some cross-reactivity in DNA vaccine antiserum when there was relatively high HA sequence similarity. For example, we observed some cross-reactivity between the H2 and H5 subtypes and between the H4 and H14 subtypes (Table 2).

In addition to sequence similarity, conventional antisera showed some cross-reactivity when there were homologous NA subtypes (Table 3). For example, we observed some cross-reactivity between the H9N2 antigen and H3N2 antiserum and also between the H5N9 antigen and H2N9 and H11N9 sera.

Western and immunodot blot assays. Western and immunodot blot assays were performed to compare the specificities of the antisera prepared by conventional and DNA vaccination. While antiserum prepared by H5-pCIneo DNA vaccine reacted strongly only to the HA1 protein of the virus, H5 antiserum prepared by the conventional method reacted to several different viral proteins (Fig. 4a).

Immunodot blot assays conducted using conventional H5 and H7 antisera showed equal reactivity of the antibody against 15 subtypes of AI strains used in this study (Fig. 4b, panel 3). However, antiserum prepared by DNA vaccination reacted strongly only to the homologous strains, although weak cross-reactivity was observed with some different subtypes (Fig. 4b, panels 1 and 2). The H7 and H15 subtypes showed the highest cross-reactivity.

TABLE 2. Cross-HI test with antiserum obtained by DNA vaccination

Antigen ^a	Antiserum titer ^b																
	H1	H2	H3	H4	H5NA	H5EA	H6	H7NA	H7EA	H8	H9	H10	H11	H12	H13	H14	H15
H1N1	128	— ^c	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H2N8	—	128	—	—	4	4	—	—	—	—	—	—	—	—	—	—	—
H3N8	—	—	128	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H4N6	—	—	—	128	—	—	—	—	—	—	—	—	—	—	—	—	—
H5N9-NA	—	—	—	—	128	32	—	—	—	—	—	—	—	—	—	—	—
H5N1-EA	—	—	—	—	64	128	—	—	—	—	—	—	—	—	—	—	—
H6N8	—	—	—	—	4	4	128	—	—	—	—	—	—	—	—	—	—
H7N2-NA	—	—	—	—	—	—	—	128	32	—	—	—	—	—	—	—	—
H7N1-EA	—	—	—	—	—	—	—	128	128	—	—	—	—	—	—	—	—
H8N4	—	—	—	—	—	—	—	—	—	128	—	—	—	—	—	—	—
H9N2	—	—	—	4	—	—	—	—	—	—	128	—	—	—	—	—	—
H10N7	—	—	—	—	—	—	—	—	—	—	—	128	—	—	—	—	—
H11N1	—	—	—	—	—	—	4	—	—	—	—	—	128	—	—	—	—
H12N5	—	8	—	—	—	—	—	—	—	—	—	—	—	128	—	—	—
H13N6	—	—	—	—	—	—	4	—	—	—	—	—	—	—	128	—	—
H14N5	—	—	—	8	—	—	—	—	—	—	—	—	—	—	—	16	—
H15N9	—	—	—	—	—	—	—	16	—	—	—	—	—	—	—	—	128

^a The same viruses used for DNA vaccine construction were used as the antigen, except for the H4 antigen, where A/duck/NJ/5406-27/94 was used. NA, North American lineage virus; EA, Eurasian lineage virus.

^b The HI titers of the sera, except H14 serum, were adjusted to 1:128 with the homologous antigen.

^c — indicates an HI titer less than 4.

DISCUSSION

Once an influenza virus is isolated, the next step in the identification process is to determine the subtype of the surface antigens HA and NA. HA is the major antigen of the virus against which neutralizing antibodies are produced (21). Furthermore, because of its pivotal role in pathogenicity (19), HA subtyping is crucial for the control and epidemiological study of the disease. Although molecular techniques such as a reverse transcription-PCR have been used to differentiate subtypes (10), the standard method for subtyping of the HA of influenza viruses is the HI test with reference antisera to the known HA subtypes. For the HI test, preparation of antiserum

of good quality is essential. Commonly, most reference antisera are made by injecting chickens with live or killed whole-virus preparations to stimulate an antibody response. Two possible problems occur with this method: safety and steric hindrance. Laboratories must be equipped with adequate bio-safety facilities to prepare the antigen and to perform the animal experiments, especially when dealing with HPAI viruses, such as the A/chicken/Scotland/59 virus, or viruses of foreign origin, such as those used in this study. Second, steric hindrance in the HI test can occur if the antiserum used for HA subtyping also contains homologous antibodies to the NA of the unknown isolate. Antibodies against NA can interfere

TABLE 3. Cross-HI test with antiserum obtained by conventional vaccination

Antigen ^a	Antiserum titer ^b														
	H1N1	H2N2	H3N2	H4N8	H5N9	H6N2	H7N3	H8N4	H9N2	H10N7	H11N9	H12N5	H13N6	H14N5	H15N6
H1N1	64	— ^c	—	—	—	—	—	—	—	—	—	—	—	—	—
H2N8	—	128	—	—	—	—	—	—	—	—	—	—	—	—	—
H3N8	—	—	64	—	—	—	—	—	—	—	—	—	—	—	—
H4N6	—	—	4	128	—	—	—	—	—	—	—	—	—	4	—
H5N9-NA	—	—	—	—	128	—	—	—	—	—	8	4	—	—	—
H5N1-EA	4	8	—	—	32	—	—	—	—	—	—	—	—	—	—
H6N8	4	—	—	—	—	128	—	—	4	—	—	4	—	—	—
H7N2-NA	—	—	—	—	—	4	64	—	—	—	—	—	—	—	4
H7N1-EA	—	—	—	—	—	—	32	—	—	—	—	—	—	—	—
H8N4	—	—	—	—	—	—	—	128	4	—	—	—	—	—	—
H9N2	—	—	4	—	—	—	—	—	128	—	—	—	—	—	—
H10N7	—	—	—	—	—	—	—	—	—	128	—	—	—	—	—
H11N1	—	—	—	—	—	—	—	—	—	—	128	—	—	—	—
H12N5	—	—	—	—	—	—	—	—	—	—	—	128	—	—	—
H13N6	—	—	4	—	—	—	—	—	—	—	—	—	128	—	—
H14N5	—	—	4	—	—	—	—	—	—	—	—	—	—	128	—
H15N9	—	—	—	—	—	—	8	—	—	—	—	—	—	—	128

^a Viruses used for DNA vaccine construction were used as the antigen, except for the H4 antigen, where A/duck/NJ/5406-27/94 was used. NA, North American lineage virus; EA, Eurasian lineage virus.

^b The HI titer of the sera were adjusted to 1:128 with the homologous antigen.

^c — indicates an HI titer less than 4.

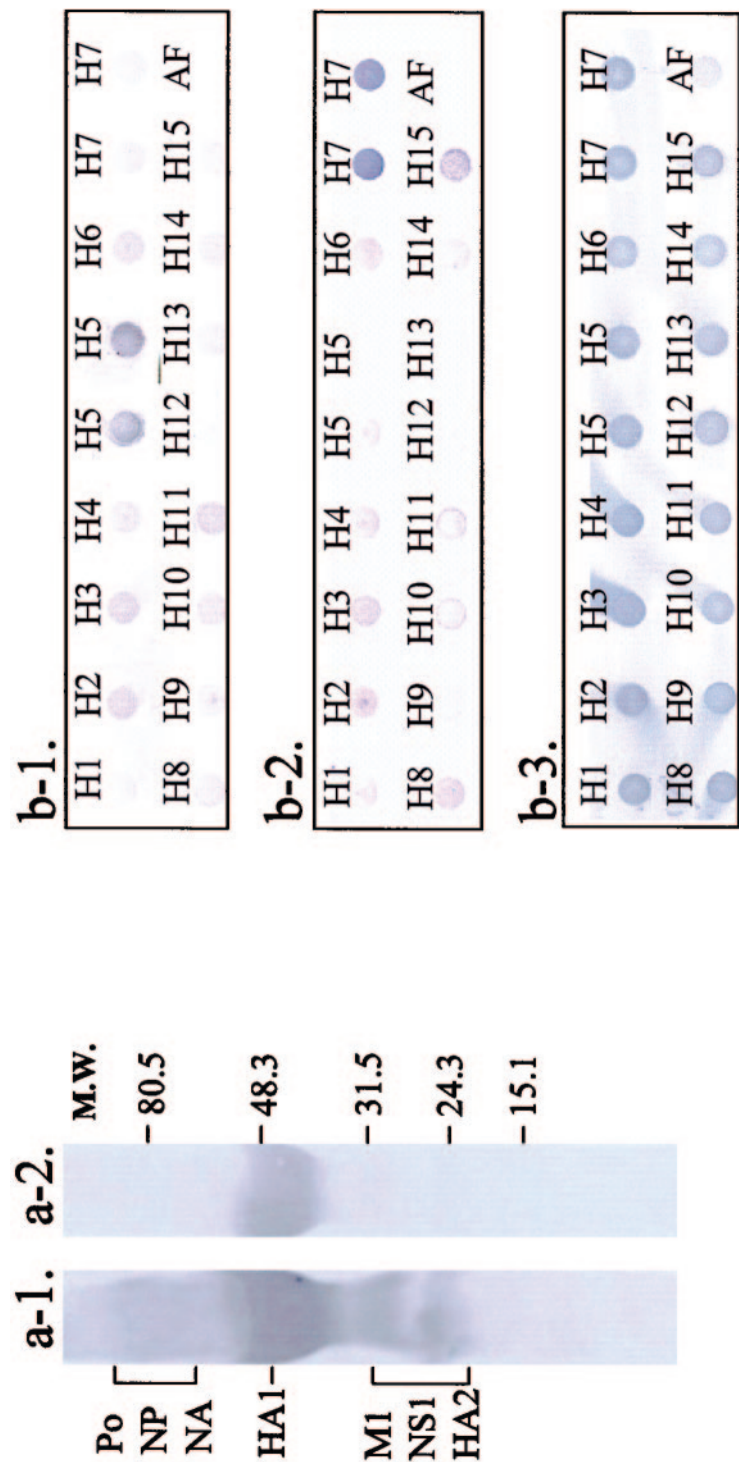


FIG. 4. Western (a) and immunodot blot (b) assays were performed to compare the specificities of the antisera prepared by conventional or DNA vaccination. (a) H5 (A/turkey/WI/68) virus was reacted with conventional H5 serum (a-1) and DNA vaccine H5 serum (a-2). (b) Viruses, used in HI test as antigens (Table 2), were dotted onto an NC membrane and reacted with DNA vaccine H5 serum (b-1), DNA vaccine H7 serum (b-2), and conventional vaccine H5 serum (b-3). M.W., molecular weight (thousands).

nonspecifically with HA, leading to nonspecific inhibition and possible misidentification of an isolate. Thus, subtyping is facilitated by using antisera that contain antibodies only to HA or antisera prepared against reassortant viruses with irrelevant NAs (4).

Our study shows that DNA vaccination can overcome those two limitations and may be a viable alternative to conventional methods of preparing antisera for diagnostic purposes. The antisera prepared by DNA vaccination compared favorably to conventionally prepared reference antisera used in the HI test for subtyping (Tables 2 and 3).

In HI tests with antisera prepared by DNA vaccination, minor cross-reactivities were observed between isolates that shared high identity in the HA protein sequence (Table 2). In contrast, when HI tests were conducted with antisera prepared by a conventional method, cross-reactivities were observed among some isolates that shared the same NA subtype as well as among isolates that shared high HA sequence homology (Table 3). Because a limited number of antigens were used in this study, it is not evident how much of an advantage DNA vaccine antiserum has in overcoming the steric hindrance with a homologous NA subtype. Nevertheless, DNA vaccination with plasmids that contain only the HA gene coding sequence should prevent this problem.

Other than as a diagnostic test, the HI test is also important and routinely used to evaluate antigenic relatedness of different strains, especially between vaccine and outbreak viruses, to assess the potential efficacy of a vaccine (8, 9). For this purpose, antisera prepared by DNA vaccination can be useful because the HI titer will primarily depend on the relatedness of the HA protein. We observed clear antigenic differences between Eurasian and North American lineages of H5 and H7 viruses in this study (Table 2).

The HA specificity of the antisera prepared by DNA vaccines was further demonstrated by Western blot and immunodot blot assays (Fig. 4). In Western blot analysis, antisera prepared by DNA vaccination reacted only with the HA protein under denaturing conditions, while conventional sera reacted to the HA as well as other viral proteins including NP and M. In the immunodot blot assay, HA subtype specificity was more clearly defined because H5 and H7 antisera reacted strongly to the H5 and H7 subtype antigens, respectively, and not to the others. As observed in cross-HI tests (Tables 2 and 3), some cross-reactivity was also observed between H7 sera and the H15 antigen in the immunodot blot assay. In previous work, the immunodot blot assay has been used by others for serotyping of the infectious bronchitis virus (14) and has been applied for the detection of influenza virus (11, 13). Thus, with more optimization, we also expect that the antisera prepared by DNA vaccines can be used for subtyping of influenza virus using the immunodot blot assay or similar methods.

Although the preparation of antiserum by DNA vaccination has many advantages, the biggest disadvantage compared to the conventional method is that it induces a lower HI immune response. Although we obtained relatively high HI titers with several subtypes of DNA vaccines, many other subtypes require further optimization to induce stronger immune responses. Since all the groups were treated equally with the same amount of DNA in the vaccine and an apparently similar

level of protein expression, as demonstrated with the immunofluorescent antibody test, we speculate that the lower antibody response in pCIneo-H14 and other DNA vaccine-treated groups may be due to the low immunogenicity of the protein itself. Alternatively, due to the posttranslational modification, it is also possible that the expressed protein retained the immunogenicity but lost some of its hemagglutination-inhibiting activity. It is also possible that the use of different adjuvants could have resulted in differences in the immune responses between birds vaccinated with DNA and birds vaccinated with inactivated influenza virus. Inactivated influenza virus by itself is not very immunogenic. However, for the conventional inactivated vaccine, oil is commonly used as an adjuvant and induces a much higher immune response. Thus, the immune response induced by DNA vaccine may be improved greatly by vaccination with a different adjuvant.

Regarding the specificity of the antisera, since the HA2 subunit of the HA molecule is more conserved among all influenza A viruses, it may also be possible to improve the specificity and reduce the minor cross-reactivity of the DNA vaccine antisera by using EEVs that express only the HA1 subunit or small antigenic epitopes of the HA gene. It remains to be determined if only the expressed HA1 protein will be as stable and immunogenic as the whole molecule.

In summary, we showed that DNA vaccination could be a viable alternative to the conventional method of preparing reference antisera, which relies on the use of live or killed whole influenza virus as the immunogen. Furthermore, the specificity of the antiserum prepared by DNA vaccination can be applied in different diagnostic and research purposes, and we also expect that DNA vaccination can be applied to produce antibodies to other influenza viral proteins.

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